JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5696–5700 Vol. 177, No. 19
0021-9193/95/$04.00+0

NOTES

Mutations in GltC That Increase *Bacillus subtilis gltA* Expression

BORIS R. BELITSKY AND ABRAHAM L. SONENSHEIN*

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received 9 June 1995/Accepted 1 August 1995

Mutants with altered forms of GltC, a positive LysR-type regulator of *Bacillus subtilis* glutamate synthase gene expression, were isolated. The mutant GltC proteins stimulated expression from the wild-type *gltA* promoter region 1.5- to 2.0-fold and from mutant promoter regions up to 80-fold. Moreover, expression of *gltA* became much less dependent on a nitrogen source-associated signal.

The GltC protein of *Bacillus subtilis*, a member of the LysR family of bacterial transcription activators (12, 23), stimulates expression of *gltA* and *gltB*, the genes that encode glutamate synthase (5). The *gltA* and *gltB* genes lie adjacent to *gltC* on the *B. subtilis* chromosome but are transcribed in the opposite direction (5) (Fig. 1). In an accompanying paper (3) we show that two dyad symmetry sequences (Box I and Box II) in the region immediately upstream of the *gltA* promoter are required for the positive regulatory activity of GltC at the *gltA* promoter; Box I is also necessary for negative autoregulation of *gltC*. Box II, overlapping the −35 region of the *gltA* promoter, is likely to be the site from which GltC interacts with RNA polymerase to stimulate *gltA* transcription. To gain further insight into GltC interaction with the *gltCA* regulatory region, we sought to identify mutations in the GltC protein that would compensate for defects in *gltA* expression caused by specific mutations in Box II.

Strains LG243A {*gltA194*::Tn*917* Δ*amyE*::[*cat* Φ(*gltC′p43-gusA*) Φ(*gltA′p43-lacZ*)]} and LG249A {*gltA194*::Tn*917* Δ*amyE*::[cat Φ(*gltC′p49-gusA*) Φ(*gltA′p49-lacZ*)]}, derivatives of strain SMY carrying the *gltA194*::Tn*917* allele at the *glt* locus and *glt Ap43-lacZ* or *gltAp49-lacZ* fusions at the *amyE* locus, form white colonies on DS agar plates (10) containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) because of low-level expression of the mutant *gltA-lacZ* fusions. (A strain carrying a wild-type fusion would form blue colonies.) The *gltAp43* and *gltAp49* mutations are A-for-T and C-for-T substitutions, respectively, at position −48 with respect to the *gltA* transcription start point (3). We isolated spontaneous mutants (one from LG243A and two from LG249A; frequency, $\sim 10^{-7}$) that formed slightly blue or blue colonies. All three mutant phenotypes were tightly linked (~95%) to the *gltA*::Tn*917* marker during transformation (9) of LG243 or LG249 (*gltA*$^+$ versions of LG243A and LG249A), indicating that the mutations were near *gltA* and might be within *gltC*. Integrative plasmids carrying different parts of the *gltCA* regulatory region and the *gltC* gene were used to map all three compensatory mutations to defined regions internal to *gltC* (Fig. 1).

To separate the *gltC* mutations from the closely linked *gltA194*::Tn*917* (GltA$^-$ Ery^r) allele, plasmid pBB559 was first integrated by a single crossover event into the chromosome of each *gltC gltA* double mutant, to create another selectable marker near *gltC*. Plasmid pBB559 is a pBluescript SK(−) (Stratagene) derivative with a *neo* cassette (Neo^r or Kan^r) (14), inserted as a blunt-ended *Pst*I-*Eco*RI fragment at the blunt-ended *Afl*III site; it also contains between its *Cla*I and *Hin*dIII sites the 0.4-kb *Cla*I-*Hin*dIII piece of chromosomal DNA from an uncharacterized region (*unk*) located 1 kb downstream of *gltC* (4). Chromosomal DNA of the Neo^r (*unk*::pBB559) transformants was isolated (10) and used to transform strain LG200 {Δ*amyE*::[*cat* Φ(*gltC′p*$^+$-*gusA*) Φ(*gltA′p*$^+$-*lacZ*)]} to neomycin resistance. The colonies that had a GltA$^+$ Ery^s phenotype (~20% of total transformants) comprised two classes. Some had the phenotype of the parent strain LG200, and others had a novel, deeper-blue phenotype which proved to be due to the presence of the *gltC* mutations (see below). In contrast to *gltC* null mutants, which are unable to grow without glutamate, these *gltC* mutants grew at the wild-type rate in minimal medium with or without added sources of glutamate (data not shown).

Chromosomal DNA of the *gltA*$^+$ *unk*::pBB559 derivatives of the three *gltC* mutants was used as a source to clone the mutant *gltC* alleles. *Pst*I-digested fragments of the chromosome were self-ligated (21) and used to transform by electroporation (8) *Escherichia coli* JM107 (24), with selection for the Amp^r and Kan^r markers of pBB559 on L agar plates with appropriate antibiotics (19). The resulting plasmids carried 5.2-kb *Cla*I-*Pst*I fragments of the *B. subtilis* chromosome, including the entire *gltC* gene and adjacent regions of DNA (4). Sequence analysis (22) of the appropriate regions of *gltC* identified the mutations as single-base substitutions replacing Pro-88, Thr-99, and Ile-160 of the GltC protein with Leu, Ala, and Lys, respectively (Fig. 2). None of these mutations altered the putative helix-turn-helix region of GltC (positions 18 to 37), apparently responsible for DNA binding (23).

All three *gltC* mutations caused a moderate increase (1.5- to 2-fold) in expression from the wild-type *gltA* promoter under activating conditions (3, 4), i.e., in liquid TSS minimal glucose medium (10) with ammonia as the sole nitrogen source (Table 1, experiment 1). However, under nonactivating conditions (3), in proline medium, the *gltC* mutations caused a much larger

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6761. Fax: (617) 636-0337. Electronic mail address: Asonensh@opal.tufts.edu.

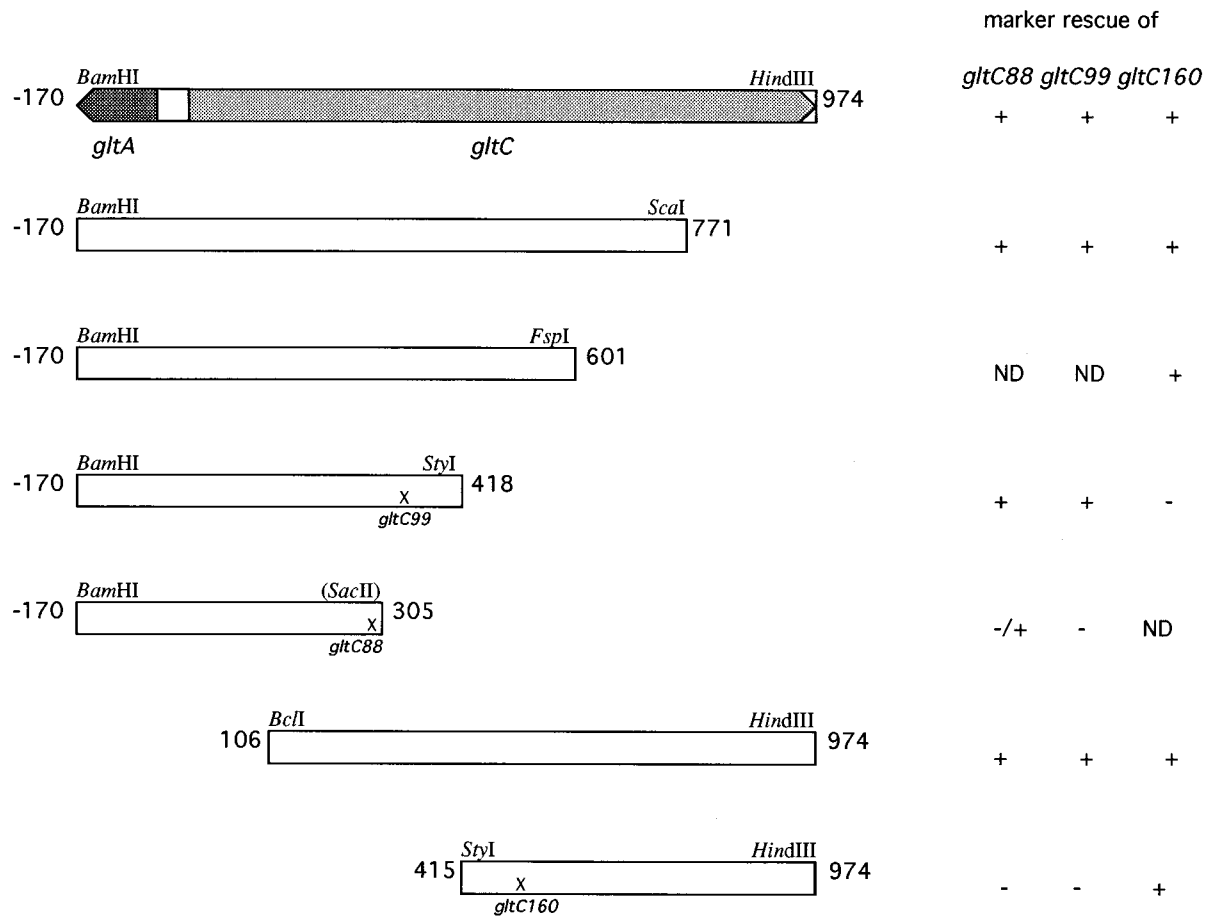

FIG. 1. Mapping of *gltC* mutations by marker rescue. Compensatory mutants of strains LG243A and LG249A were transformed (9) with pIPC10 (3) and its deletion derivatives (2) carrying parts of the *gltCA* regulatory region and the *gltC* gene. The boundaries of the *gltCA* fragments are given with respect to the proposed *gltC* transcription initiation site (3, 5). The *Sac*II site (in parentheses) was constructed by oligonucleotide-directed mutagenesis (15). X, positions of *gltC* mutations. The + and − notations indicate the ability of plasmids to rescue mutations in the *gltC* gene (a −/+ notation reflects very low efficiency of marker rescue). Compensatory mutations confer a blue-colony phenotype on the parent strains; after marker rescue, transformants regain the white-colony phenotype. ND, not determined.

(15- to 30-fold) increase in expression. As a result, the activation ratio (that is, *gltA-lacZ* expression in ammonia medium versus proline medium) was reduced from >120-fold in wild-type cells to 8- to 14-fold in *gltC* mutant cells (Table 1, experiment 1).

In addition to serving as a positive regulator of *gltA* expression, GltC is a negative autoregulator (3, 5). The *gltC99* mutation decreased expression of a *gltC-gusA* fusion seven- to eightfold (*gltC-gusA* expression presumably reflects the level of the GltC-T99A [GltC with a T-to-A change at position 99] protein in the cells, and thus the potential of this mutant protein to activate *gltA* expression may be underestimated). The other two *gltC* mutations had small effects on *gltC-gusA* expression (Table 1, experiment 1).

As expected from the original mutant isolation scheme, the *gltC* mutations compensated for the deleterious effects on *gltA-lacZ* expression of some mutations in the *gltCA* regulatory region (Fig. 3). None of the mutant proteins was a specific suppressor of any single mutation or of mutations only in Box II. The GltC-I160K protein, selected as a compensator of the T(−48)C mutation in Box II, increased expression of all mutant fusions to about the same moderate extent (1.4- to 3.6-fold) as for the wild type fusion, i.e., it did not compensate specifically for any particular defect in the *gltCA* regulatory region. The GltC-P88L and GltC-T99A proteins, selected as compensators of T(−48)A and T(−48)C mutations, respectively, compensated very efficiently for some (but not the same) regulatory region defects and had little or no effect on utilization of other mutant regulatory regions (Fig. 3).

The fact that different mutations in GltC affect expression of various versions of the *gltCA* regulatory region according to very different patterns and to varying extents (Fig. 3D) strongly suggests that stimulation of *gltA* transcription involves direct interaction of GltC with the Box I-Box II regulatory region, rather than any modulating effect of GltC on the synthesis or activity of some other protein.

It is puzzling, though, why the three mutant GltC proteins, which interact so differently with various mutant forms of the *gltCA* regulatory region, show such similar alterations in the response to nitrogen sources. The basis for apparent partial effector independence of GltC may be indirect, however. Mutations that increase by about 1 helical turn the separation of Box I and Box II in the *gltCA* regulatory region also increase *gltA* expression and cause partial relief of nitrogen source dependency in cells containing wild-type GltC (3). The most likely explanation for these phenotypes is that the insertion mutations facilitate interaction of GltC proteins (most probably dimers) bound to Box I and Box II (3). The fact that such spacing mutations and the *gltC* compensatory mutations have very similar effects and are not additive when combined in the

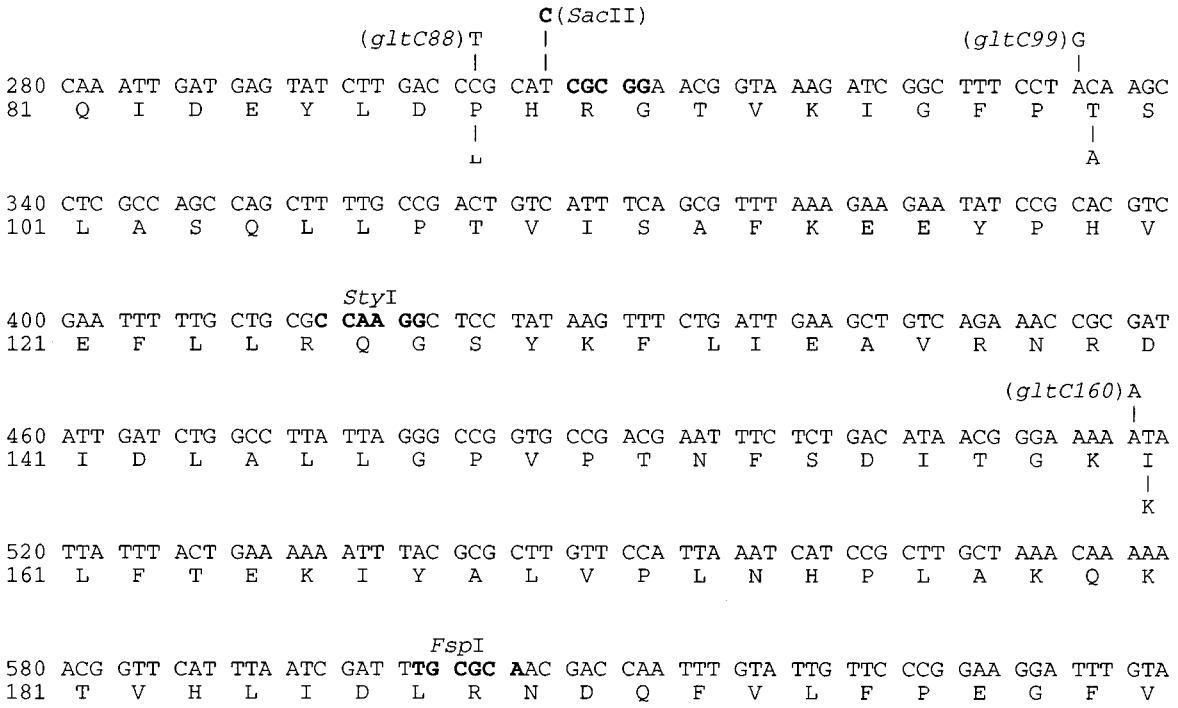

FIG. 2. Sequence alterations in *gltC* mutants. The sequences of the internal parts of the *gltC* gene and GltC protein are shown. Nucleotide and amino acid changes are shown above and below the sequence lines, respectively. Coordinates refer to the proposed *gltC* transcription initiation start point and GltC codon positions, respectively (3, 4). Relevant restriction sites are shown in boldface type. The *Sac*II site (in parentheses) was constructed by oligonucleotide-directed mutagenesis (15) replacing T (position 306) with C. The mutations were sequenced by the dideoxy chain termination method (22) with *glt*-specific oligonucleotides. Plasmid double-stranded DNA to be sequenced was purified by using the Qiagen miniprep purification kit (Promega Corp.). A Sequenase reagent kit (United States Biochemical Corp.) was used according to the protocol of the manufacturer. The wild-type sequence has been assigned GenBank accession number M28509.

TABLE 1. Effects of *gltC* mutations on *gltA-lacZ* and *gltC-gusA* expression

gltC allele (at the *glt* locus)	Regulatory region within fusion (at the *amyE* locus)	β-Galactosidase activity[a] (*gltA-lacZ* fusion)			β-Glucuronidase activity[a] (*gltC-gusA* fusion)	
		NH_4Cl	Pro	NH_4Cl/Pro ratio	NH_4Cl	Pro
Expt 1[b]						
gltC$^+$	*gltAp*$^+$	75.8	<0.6	>120	0.7	0.8
gltC88	*gltAp*$^+$	110.8	12.5	8.9	1.5	1.2
gltC99	*gltAp*$^+$	154.1	19.9	7.7	0.1	0.1
gltC160	*gltAp*$^+$	114.5	8.2	14.0	0.5	0.5
Expt 2[c]						
gltC$^+$	*gltAp86*	183.5	23.3	7.9	0.1	0.1
gltC88	*gltAp86*	54.1	ND[d]	ND[d]	1.9	ND[d]
gltC99	*gltAp86*	39.2	ND[d]	ND[d]	3.2	ND[d]
gltC160	*gltAp86*	152.3	19.7	7.7	0.1	0.1

[a] Bidirectional *gltA′-lacZ* and *gltC′-gusA* fusion constructs were at the *amyE* locus (3). Cells were grown in TSS-glucose minimal medium with different nitrogen sources (10). β-Galactosidase and β-glucuronidase activities were assayed as described previously and expressed in units (3). The averages of at least duplicate experiments are presented (variance was less than 15%).

[b] Strain LG200 (3) and its *unk*::pBB559 *gltC* derivatives (*unk*::pBB559 had no effect on *gltA* and *gltC* expression [data not shown]).

[c] *gltAp86* is a mutant version of the *gltCA* regulatory region in which the distance between Box I and Box II is increased by 9 bp (3). The fusion was integrated into strain SMY (*glt*$^+$) or its *unk*::pBB559 *gltC* derivatives.

[d] ND, not determined.

same cell (Table 1, experiment 2) is consistent with the idea that they affect the same aspect of *gltA* activation, that is, GltC dimer-dimer interaction. Moreover, the decreased activity of the *gltAp86-lacZ* fusion (with increased spacing between Box I and Box II) in *gltC88* and *gltC99* mutants suggests that these mutant GltC proteins are not simply locked in a partially activated conformation (Table 1, experiment 2). To rationalize our results, we speculate that the modified response to a nitrogen source-dependent signal is the result of altered dimer-dimer interactions of the compensatory GltC proteins.

The GltC-P88L protein stimulates expression from the *gltA-lacZ* fusion containing the Ω(−57, −56)A mutation nearly to the level obtained with the wild-type fusion (Fig. 3A), even though this insertion alters both the distance between Box I and Box II and their relative rotational orientation. This result can best be explained by creation of novel contacts between dimers of GltC-P88L. The contacts made by GltC-T99A are still dependent on the precise rotational orientation of Box I and Box II (Fig. 3B), but they seem to be partially disrupted (as are GltC-P88L dimer-dimer contacts) if the distance between the two boxes is increased by 1 helical turn (Table 1). When Box I and Box II are in their normal positions, the contacts formed by these mutant proteins must be stronger than those made by wild-type GltC, since they partially alleviate the requirement that both boxes be intact. The sequence of Box II is particularly malleable in the *gltC99* mutant (Fig. 3). Such altered contacts may also be more effective at the wild-type promoter, leading to the elevated expression and partial constitutivity with respect to the nitrogen source seen with these mutants.

The I160K mutation increases expression from wild-type and from all Box I and Box II mutant *gltA* promoters to the

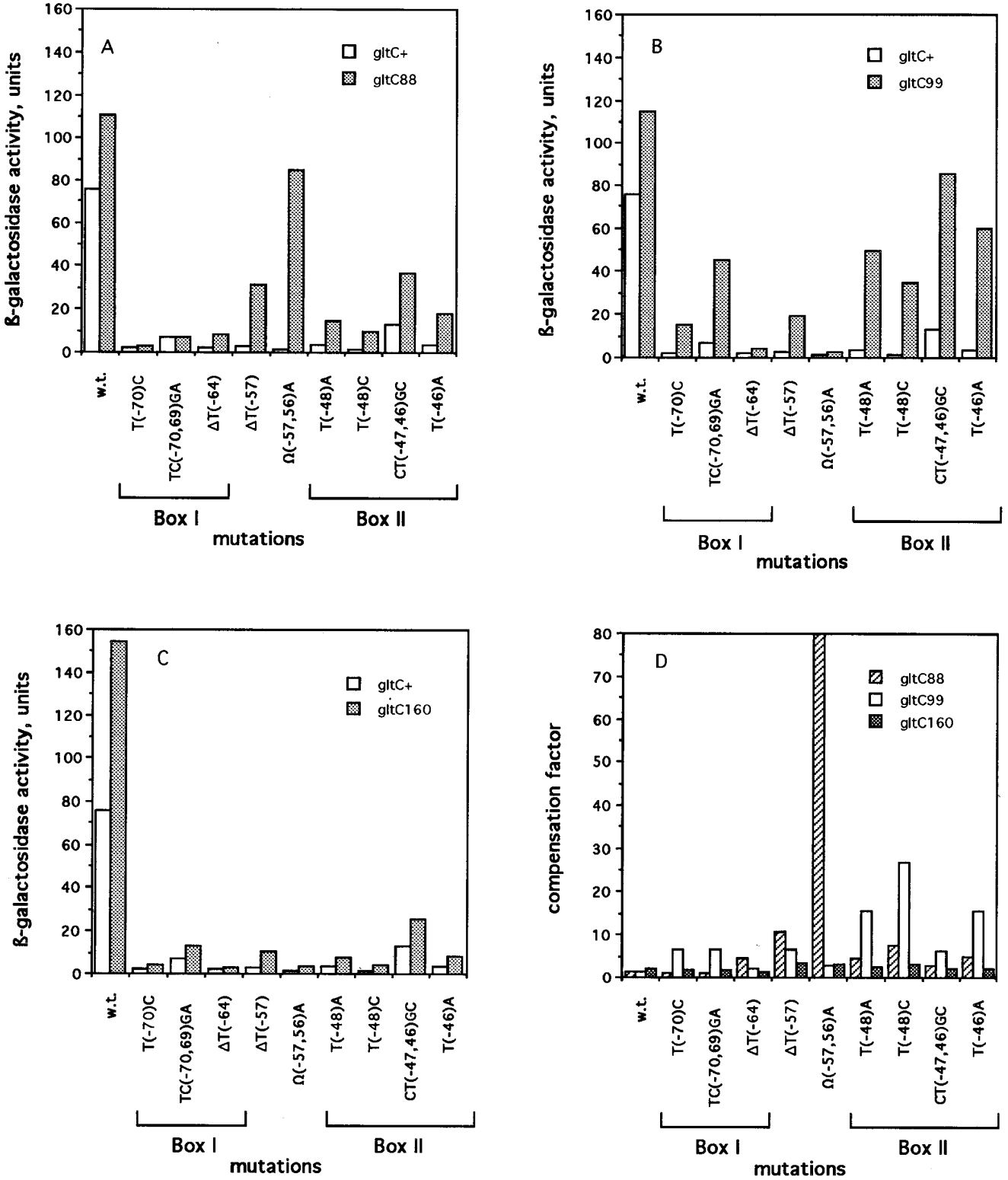

FIG. 3. Effect of mutations in *gltC* on *gltA-lacZ* expression. *B. subtilis* strains carrying compensatory mutations in *gltC* (together with the *unk*::pBB559 insertion) and *gltA-lacZ* fusions with different mutations in the regulatory region integrated at the *amyE* locus were grown at 37°C in TSS (10) minimal medium with 0.5% glucose and 0.2% NH_4Cl as sole carbon and nitrogen sources, respectively. β-Galactosidase activity was assayed as described previously (3). (A) *gltC88*; (B) *gltC99*; (C) *gltC160*; (D) ratio between activities of individual *gltA-lacZ* fusions in a *gltC*$^+$ strain and in each of the *gltC* mutants (compensation factor). w.t., wild type.

same relative extent. The increased expression from the wild-type promoter is slightly enhanced by a 1-helical-turn insertion between Box I and Box II (Table 1). We surmise that interaction between GltC-I160K dimers is essentially normal but more effective than interaction of wild-type dimers, thereby again causing partial effector independence.

In summary, we suggest that the function of a nitrogen source-dependent signal in *gltA* regulation is to promote GltC dimer-dimer interaction as a means of facilitating or stabilizing GltC binding to Box II (the activation site). Any mutation in GltC that leads to stronger dimer-dimer interaction (as postulated here for the compensatory *gltC* mutations) or tighter binding to Box II should alleviate the requirement for a nitrogen source-dependent signal in *gltA* activation.

Stronger dimer-dimer contacts not only may stabilize GltC binding to Box II but also could be expected to increase GltC binding to Box I. This should be reflected in a higher level of repression of a *gltC-gusA* fusion, as was observed for the GltC-T99A protein (Table 1, experiment 1). But in some cases, stronger binding to Box II may displace the GltC protein complex in such a way that it partially loses its ability to autorepress. This may explain why we do not see a correlation between abilities of the mutant GltC proteins to activate *gltA* and repress *gltC* (Table 1).

All three *gltC* compensatory mutants have substitutions in or near a region in LysR-type proteins that has been thought to be involved in effector recognition and response (11, 23), because mutants altered in this region (defined by positions 95 to 173 in various proteins) either acquire partial activity in the absence of their effectors or fail to respond to the effectors, even though they are not altered in DNA binding (23). Partially constitutive mutants, similar to those described here for GltC, have been identified for NodD (18), CatM (20), NahR (13), CysB (7, 16), AmpR (1), OccR (6), and OxyR (17) proteins. It seems possible that the apparent defects in effector response attributed to mutant forms of these proteins are actually due to changes in protein-protein interaction.

We thank A. Wright for careful reading of the manuscript.
This work was supported by Public Health Service grant GM36718.

REFERENCES

1. **Bartowsky, E., and S. Normark.** 1993. Interaction of wild-type and mutant AmpR of *Citrobacter freundii* with target DNA. Mol. Microbiol. **10:**555–565.
2. **Belitsky, B. R.** Unpublished data.
3. **Belitsky, B. R., P. J. D. Janssen, and A. L. Sonenshein.** 1995. Sites required for GltC-dependent regulation of *Bacillus subtilis* glutamate synthase expression. J. Bacteriol. **177:**5686–5695.
4. **Bohannon, D. E., M. S. Rosenkrantz, and A. L. Sonenshein.** 1985. Regulation of *Bacillus subtilis* glutamate synthase genes by the nitrogen source. J. Bacteriol. **163:**957–964.
5. **Bohannon, D. E., and A. L. Sonenshein.** 1989. Positive regulation of glutamate biosynthesis in *Bacillus subtilis*. J. Bacteriol. **171:**4718–4727.
6. **Cho, K., and S. C. Winans.** 1993. Altered-function mutations in the *Agrobacterium tumefaciens* OccR protein and in an OccR-regulated promoter. J. Bacteriol. **175:**7715–7719.
7. **Colyer, T. E., and N. M. Kredich.** 1994. Residue threonine-149 of the *Salmonella typhimurium* CysB transcription activator: mutations causing constitutive expression of positively regulated genes of the cysteine regulon. Mol. Microbiol. **13:**797–805.
8. **Dower, W. J., F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *Escherichia coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
9. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. J. Mol. Biol. **56:**209–221.
10. **Fouet, A., and A. L. Sonenshein.** 1990. A target for carbon source-dependent negative regulation of the *citB* promoter of *Bacillus subtilis*. J. Bacteriol. **172:**835–844.
11. **Györgypal, Z., and A. Kondorosi.** 1991. Homology of the ligand-binding regions of *Rhizobium* symbiotic regulatory protein NodD and vertebrate nuclear receptors. Mol. Gen. Genet. **226:**337–340.
12. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
13. **Huang, J., and M. A. Schell.** 1991. *In vivo* interactions of the NahR transcriptional activator with its target sequences. Inducer-mediated changes resulting in transcription activation. J. Biol. Chem. **266:**10830–10838.
14. **Itaya, M., K. Kondo, and T. Tanaka.** 1989. A neomycin resistance cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. **17:**4410.
15. **Janssen, P. J. D.** Unpublished data.
16. **Kredich, N. M.** 1992. The molecular basis for positive regulation of *cys* promoters in *Salmonella typhimurium* and *Escherichia coli*. Mol. Microbiol. **6:**2747–2753.
17. **Kullik, I., M. B. Toledano, L. A. Tartaglia, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J. Bacteriol. **177:**1275–1284.
18. **McIver, J., M. A. Djordjevic, J. J. Weinman, G. L. Bender, and B. G. Rolfe.** 1989. Extension of host range of *Rhizobium leguminosarum* bv. *trifolii* caused by point mutations in *nodD* that result in alterations in regulatory function and recognition of inducer molecules. Mol. Plant-Microbe Interact. **2:**97–106.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Neidle, E. L., C. Hartnett, and L. N. Ornston.** 1989. Characterization of *Acinetobacter calcoaceticus catM*, a repressor gene homologous in sequence to transcriptional activator genes. J. Bacteriol. **171:**5410–5421.
21. **Sambrook, J., E. F. Fritsch, and T. J. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
23. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
24. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.